Guo et al., Afr J Tradit Complement Altern Med., (2018) 15 (1): 1-10
https://doi.org/10.21010/ajtcam.v15i1.1

METABOLIC CHANGES INDUCED BY BUSHENHUOXUE GRANULES ON STRIATUM AND SUBSTANTIA NIGRA IN A RAT MODEL OF PARKINSON'S DISEASE

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Article History

Received: Dec. 13, 2016

Revised Received: Sept. 5, 2017

Accepted: Sept. 5, 2017

Published Online: Dec. 29, 2017

Abstract

Background: Parkinson's disease is a neurodegenerative disease, while its mechanism is still unclear. Long-term levodopa-based treatment leads to decreased response or loss of response, as well as severe side effects. Our previous study has proved that Bushenhuoxue Granules have effects on Parkinson's disease, but the underlying mechanism is still need to be explored. Our research is to investigate the mechanisms of Bushenhuoxue Granules on Parkinson's disease (PD) by examining changes in the expression of the adenosine A_{2A} receptor、vesicular monoamine transporter 2 (VMAT2)、divalent metal transporter 1（DMT1） and nuclear factor E2 related (Nrf2) in a rat model of Parkinson's disease (PD) .
Materials and Methods: Changes in the apomorphine (APO)-induced rotational behavior of rats were observed after treatment. Immunofluorescence and immunohistochemistry were performed to investigate changes in adenosine A_{2A} receptor 、VMAT2、DMT1 and Nrf2 expression in the rat striatum and substantia nigra.
Results: Rotations after treatment were199.11 ± 27.16, which significantly decreased compared with that before treatment (273.0 ± 44.61, $p < 0.01$). Adenosine A_{2A} receptor expression in the striatum was 3.10 ± 0.34 significantly increased in the model group and decreased in the normal control group, whereas the expression level in the Bushenhuoxue group was 1.13 ± 0.23,$p < 0.05$ between the two control groups. No adenosine A_{2A} receptor expression was observed in the substantia nigra. VMAT2 expression in the rat striatum was 23.20 ± 2.68 and substantia nigra was 15.98 ± 0.70 increased in the normal control group. They were 8.99 ± 0.48 in the rat striatum and 8.45 ± 0.59 substantia nigra significantly decreased in the model control group, whereas the expression level in the Bushenhuoxue group was 15.36 ± 0.89 in the rat striatum and 11.69 ± 1.17 in the rat substantia nigra ($p < 0.05$), also between the two control groups. DMT1 expression in the rat striatum was 3.30 ± 0.30 and substantia nigra was 6.56 ± 0.64 decreased in the normal control group. They were 7.92 ± 0.52 in the rat striatum and 12.76 ± 0.86 substantia nigra significantly increased in the model control group, whereas the expression level in the Bushenhuoxue group was 6.17 ± 0.27 in the rat striatum and 9.13 ± 0.44 in the rat substantia nigra ($p < 0.05$), also between the two control groups. Nrf2 expression in the rat striatum was 7.90 ± 0.29 and substantia nigra was 15.22 ± 1.22 increased in the normal control group. They were 3.09 ± 0.43 in the rat striatum and 8.57 ± 0.54 substantia nigra significantly decreased in the model control group, whereas the expression level in the Bushenhuoxue group was 5.00 ± 0.34 in the rat striatum and 12.46 ± 0.62 in the rat substantia nigra ($p< 0.05$), also between the two control groups.
Conclusion: Bushenhuoxue Granules significantly improved the rotational behavior of PD's rats, decreased adenosine A_{2A} receptor expression, and increased VMAT2 expression; decreased DMT1 expression, and increased Nfr2 expression.

Keywords: Parkinson's disease; Traditional Chinese Medicine; Bushenhuoxue Granules; Striatum and Substantia nigra; Rats.

Introduction

The etiology of Parkinson's disease (PD) is unknown, and effective treatments are not yet available. Long-term levodopa-based treatment leads to decreased response or loss of response, and the treatment is associated with side effects of varying severity (Fabbri *et al.* 2017). Bushenhuoxue Granules, which is the Chinese herbal compound mainly used for treating PD and had been investigated in preliminary studies in early and advanced Parkinson's disease. PD is closely related to the adenosine A_{2A} receptor、vesicular monoamine transporter 2 (VMAT2)、divalent metal transporter 1(DMT1) and Nuclear factor E2 related factor2(Nrf2)(Mori A.2014; Brighina *et al.* 2013; Meng *et al.* 2017). The purpose of our study is to compare rotational behavior and changes in adenosine A_{2A} receptor、VMAT2、DMT1 and Nrf2 expression in a rat model of PD . In this study, we established a PD rat model, observed changes in rotational behavior of the PD rats after treatment, investigated changes in adenosine A_{2A} receptor VMAT2、DMT1 and Nrf2 expression in the rat striatum and substantia nigra, and explored potential effects of Bushenhuoxue Granules on PD.

Materials and Methods

Experimental animals

A total of 120 specific-pathogen-free male Sprague-Dawley (SD) rats, weighing 190 to 210 g, were provided by Beijing Vital River Laboratory Animal Technology Co, Ltd. (license number: SCXK [Beijing] 2012-0001). The rats were maintained at room temperature (20-22°C) for 2 weeks with 12 hours of continuous light and 12 hours of continuous dark per 24 hours and free access to water and food. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the local animal care and use committee.

Reagents

Six-hydroxydopamine (6-OHDA; Sigma Inc., USA), apomorphine (APO) (Shenyang First Pharmaceutical Factory), A_{2A} antibody (Millipore Corp., USA), and VMAT2 antibody (Abcam Plc., UK); DMT1 antibody (Abcam Plc., UK), and Nrf2 antibody (AbcamPlc., UK) were used in this study.

Chinese herbal compound

Bushenhuoxue Granules consisted of dogwood 20 g, cistanche 10 g, Polygonum 15 g, chuan xiong 10 g, angelica 10 g, Salvia 15 g, and 2 centipedes and were prepared by the Chinese PLA General Hospital, PLA Institute of Traditional Chinese Medicine (lot number 100 101; 8 g/bag). Using a can for extraction of traditional Chinese medicines, we decocted the mixture twice under a normal temperature and pressure condition, each time lasting 2 h. Then, we added water 10 times into the extracts, and mixed the two extracts. The above extracts were further concentrated into concrete with a vacuum recycling evaporator under a low temperature (60-65℃) condition. Then, the concrete was sprayed, dried, and grained by a spraying and drying granulator. For rats, 10 times of the normal dose for a 70kg adult was used. Bushenhuoxue Granules were dissolved in saline and administered daily via gavage at 9:00-10:00 am for 8 weeks. The quality of above herbal medicines was controlled according to Chinese Pharmacopoeia.

Animal groups

A total of 120 rats were randomly assigned with a randomization number sheet into the normal control group (the normal group; n = 20) and the model group (n = 100). The model group was further randomly assigned using a randomization number sheet into the operation group (n = 85) and the sham operation group (n = 15).

Establishment of the rat model

SD rats were housed in the Animal Center of Chinese PLA General Hospital for two weeks to acclimate the rats and establish routine maintenance. Once confirmed to have no rotational behavior, the rats were used to establish a PD rat model. The rats fasted for 12 hours before the operation and received humane care according to the 3R (Replacement, Reduction and Refinement) principle of animal use. The 85 rats in the operation group were weighed and numbered and received anesthesia with intraperitoneal injection of 6% chloral hydrate (350 mg/kg); the rats were then secured on a stereotaxic instrument in a prone position. The cranial area was sterilized, and the hair was shaved; the skin and fascia were then incised along the median sagittal line to expose the skull. Then, the incisor bar was adjusted so the front fontanelle and rear fontanelle were in the same plane. According to the Rat Brain Stereotaxic Atlas (Bao and Shu 1991), the right medial forebrain bundle was located and a needle was inserted 7.6 mm deep at 4.3 mm posterior to the front fontanelle and 1.5 mm to the right of the sagittal line. A dental drill was used to drill through the skull, and a micro-injection pump was used to

draw up 8 μL of 6-OHDA and inject 6-OHDA at 1 μL/min. The needle was left for 5 minutes after each injection before being removed slowly. Povidone-iodine was used for local sterilization, and the scalp was sutured. The 20 rats in the normal control group were not treated.

Rotational behavior test

Rotational behavior was tested at two weeks after the operation. First, after the shackles were put on, the rats were acclimated in the rotation detector for approximately 5 minutes. Once the rats calmed down, they received subcutaneous injections of APO (0.5 mg/kg). The rats usually started to exhibit rotational behavior within 2 to 4 minutes after injection. PD rats exhibited left and right (especially left) lateral rotation in a head-to-tail state. Five minutes after injection, the rotation detector started to record the number of rotations for 30 minutes. The change in the rotational behavior of the rats was observed and recorded. For the rotational behavior of the rats, the number of rotations was calculated as the total left (non-injury side) rotations minus the total right (injury side) rotations; if the number of rotations was > 210 rotations/30 minutes, the PD rat model was successfully established.

Dosing

The 54 successfully established PD rat models were randomly assigned with a randomization number sheet into two groups: the Bushenhuoxue group (the treatment group; n = 30) and the model control group (the control group; n = 24). According to the rat-human dose conversion formula (Shi 1986), the dose per kg of rat should be 10-fold that of an adult human (60 kg). Bushenhuoxue Granules were dissolved in saline and administered daily via gavage at 9:00-10:00 am for 8 weeks. The rats were weighed every week, and the dose was adjusted based on the weight. The rats in the normal group and the control group were administered saline (weight-adjusted) every day with the same gavage method and the same dose conversion method for saline as those used for the treatment group.

Sample collection and processing

Normal and model rats (after test of rotational behavior) were quickly sacrificed by decapitation. The brain tissue was collected and placed on a foil-lined tray. The tray was placed flat on liquid nitrogen to snap-freeze the tissue. The tissue was then removed and stored in a -80°C freezer. The tissues were sectioned (approximately 10 μm) with a cryostat.
Hematoxylin and eosin (HE) staining of the substantia nigra and immunofluorescent and immunohistochemical detection of adenosine A_{2A} receptor 、VMAT2、DMT1 and Nrf2 in the rat striatum and substantia nigra were performed in strict accordance with the manufacturer's instructions. The Leica QWin V3 image analysis system was used to analyze the immunohistochemical staining of adenosine A_{2A} receptor 、VMAT2、DMT1 and Nrf2 in the rat brains.

Statistical analysis

SPSS17.0 software was used for the statistical analysis. The measurement data are expressed as the means ± standard deviations. A t-test was performed for between-group comparisons. One-way repeated measures analysis of variance was performed for among-group comparisons. $P < 0.05$ was considered significant.

Results

Rotational behavior of the PD rats

After gavage, APO-induce rotations were observed for 30 minutes. The rotations were significantly reduced after treatment. ($p < 0.01$).(Table 1)

Table 1: Rotations before and after treatment in the two groups of PD rats (x ± s)

Group	n	Rotations before treatment	Rotations after treatment
Treatment	30	273.0 ± 44.61	199.11 ± 27.16 *△
Control	24	277.53 ± 44.28	275.73 ± 45.47

Note: * $p < 0.01$, before treatment vs after treatment in the treatment group; △ $p < 0.01$, the treatment group vs the control group after treatment.

Adenosine A_{2A} receptor expression in the rat striatum and substantia nigra

Immunofluorescence showed that adenosine A_{2A} receptor (red) was expressed in the cytoplasm and cell membrane in the striatum. Adenosine A_{2A} receptor expression was low in the striata of the normal group and was significantly increased in the control group, whereas the expression level in the treatment group was between the normal group and the control group (Figure 1). No adenosine A_{2A} receptor expression was observed in the rat substantia nigra.

VMAT2 expression in the rat striatum and substantia nigra

Immunofluorescence showed that VMAT2 (green) was expressed in the cytoplasm of the striatum. VMAT2 expression was high in the striata of the normal group and was significantly decreased in the control group, whereas the expression level in the treatment group was between the normal group and the control group (Figure 1).

Immunohistochemistry showed that VMAT2 was expressed in the cytoplasm of the substantia nigra; under light microscopy, nuclei stained blue, and the cytoplasm was stained brownish yellow (positive expression). VMAT2 expression was high in the striata of the normal group and was significantly decreased in the control group, whereas the expression level in the treatment group was between the normal group and the control group (Figure 2).

DMT1 expression in the rat striatum and substantia nigra

Immunofluorescence showed that DMT1 (green) was expressed in the cytoplasm and nucleus in the striatum. DMT1expression was low in the striata of the normal group and was significantly increased in the control group, whereas the expression level in the treatment group was between the normal group and the control group (Figure 1)

Immunohistochemistry showed that DMT1 was expressed in the cytoplasm of the substantia nigra; under light microscopy, nuclei stained blue, and the cytoplasm was stained brownish yellow (positive expression). DMT1 expression was low in the striata of the normal group and was significantly increased in the control group, whereas the expression level in the treatment group was between the normal group and the control group. (Figure 2)

Nrf2 expression in the rat striatum and substantia nigra

Immunofluorescence showed that *Nrf2* (green) was expressed in the cytoplasm of the striatum; under light microscopy, nuclei stained green. *Nrf2* expression was high in the striatum of the normal group and was significantly decreased in the control group, whereas the expression level in the treatment group was between the normal group and the control group. (Figure 1)

Immunohistochemistry showed that *Nrf2* was expressed in the cytoplasm and nucleus of the substantia nigra ; under light microscopy, nuclei stained blue, and the cytoplasm was stained brownish yellow (positive expression). *Nrf2* expression was high in the substantia nigra of the normal group and was significantly decreased in the control group, whereas the expression level in the treatment group was between the normal group and the control group. (Figure 2)

HE staining of substantia nigra in rats of each group (Figure 2)

Under light microscope, the structure of substantia nigra of the Normal rats was clear with normal structure basically. The nuclei of neurons are large and round with uniform staining inside,and the nucleus membrane is of integrity with visible membrane gap. The volume of the neuronal cell of the model rats get decreased and the structure is not clear and complete with fewer membrance gaps. Comparatively, the observation of the treatment group is between the other groups.

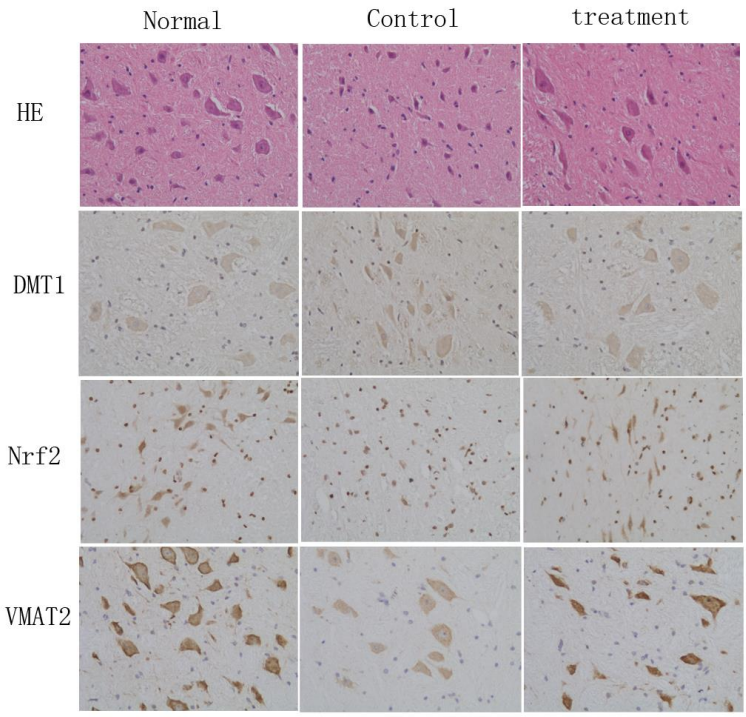

Figure 1: Adenosine A_{2A} receptor expression in the rat striatum

Immunofluorescence showed that adenosine A_{2A} receptor (red) expression was low in the striata of the normal group and was significantly increased in the control group. The expression level in the treatment group was between the normal group and the control group. Cell nuclei is in blue. Standard bar is 65 micrometer.VMAT2 expression in the rat striatum. Immunofluorescence showed that VMAT2 (green) was high in the striata of the normal group and was significantly decreased in the control group. The expression level in the treatment group was between the normal group and the control group. Cell nuclei is in blue. Immunofluorescence showed that DMT1 (green) was expressed in the cytoplasm and nucleus in the striatum. DMT1expression was low in the striata of the normal group and was significantly increased in the control group, whereas the expression level in the treatment group was between the normal group and the control group.

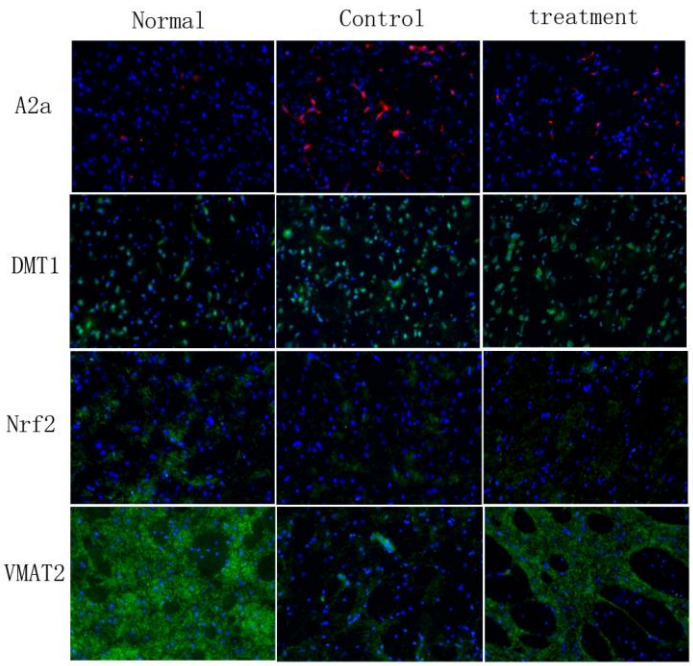

Figure 2: HE staining of substantia nigra in rats of each group

Under light microscope, the structure of substantia nigra of the Normal rats was clear with normal structure basically.The nuclei of neurons are large and round with uniform staining inside,and the nucleus membrane is of integrity with visible membrane gap. The volume of the neuronal cell of the model rats get decreased,and the structure is not clear and complete with fewer membrance gaps. Comparatively, the observation of the treatment group is between the other Two Groups.

No adenosine A_{2A} receptor expression was observed in the rat substantia nigra. Immunohistochemistry of VMAT2 in the rat substantia nigra. Nuclei stained blue, and the cytoplasm was stained brownish yellow (positive expression). VMAT2 expression was high in the substantia nigra of the normal group and was significantly decreased in the control group. The expression level in the treatment group was between the normal group and the control group. Immunohistochemistry showed that DMT1 was expressed in the cytoplasm of the substantia nigra; under light microscopy, nuclei stained blue, and the cytoplasm was stained brownish yellow (positive expression). DMT1 expression was low in the substantia nigra of the normal group and was significantly increased in the control group, whereas the expression level in the treatment group was between the normal group and the control group. Immunohistochemistry showed that *Nrf2* was expressed in the cytoplasm and nucleus of the substantia nigra; under light microscopy, nuclei stained blue, and the cytoplasm was stained brownish yellow (positive expression). *Nrf2* expression was high in the substantia nigra of the normal group and was significantly decreased in the control group, whereas the expression level in the treatment group was between the normal group and the control group. Standard bar is 65 micrometer.

Adenosine A_{2A} receptor, VMAT2, DMT1, Nrf 2-positive areas in the rat striatum

Adenosine A_{2A} receptor expression was low in the rat striatum in the normal group, whereas the expression in the treatment group was higher than the normal group and lower than the control group. VMAT2 expression was high in the rat striata in the normal group, whereas the expression in the treatment group was lower than the normal group and higher than the control group. DMT1 expression was low in the rat striatum in the normal group, whereas the expression in the treatment group was higher than the normal group and lower than the control group. Nrf2 expression was high in the rat striata in the normal group, whereas the expression in the treatment group was lower than the normal group and higher than the control group. Standard bar is 65 micrometer. (Table 2).

Table 2: Percentage of A_{2A}、VMAT2、DMT1、Nrf2-positive areas in the rat striatum ($\bar{x} \pm s$)

Group	n	A_{2A}	VMAT2	DMT1	Nrf2
Normal group	20	0.32±0.10	23.20±2.68	3.30±0.30	7.90±0.29
Control group	24	3.10±0.34	8.99±0.48	7.92±0.52	3.09±0.43
Treatment group	30	1.13±0.23*△	15.36±0.89*△	6.17±0.27*△	5.00±0.34*△

Note：* $p < 0.05$ the treatment group vs the Normal group;△$p < 0.05$，the treatment group vs the control group

Adenosine A_{2A} receptor, VMAT2, DMT1, Nrf2-positive areas in the rat substantia nigra

No adenosine A_{2A} receptor expression was observed in the rat substantia nigra. Immunohistochemistry of VMAT2 in the rat substantia nigra. Nuclei stained blue, and the cytoplasm was stained brownish yellow (positive expression). VMAT2 expression was high in the striata of the normal group and was significantly decreased in the control group. The expression level in the treatment group was between the normal group and the control group. DMT1 expression was low in the rat striatum in the normal group, whereas the expression in the treatment group was higher than the normal group and lower than the control group. Nrf2 expression was high in the rat striata in the normal group, whereas the expression in the treatment group was lower than the normal group and higher than the control group. Standard bar is 65 micrometer. (Table 3).

Table 3: Percentage of A_{2A}、VMAT2、DMT1、Nrf2-positive areas in the rat substantia nigra ($\bar{x}$ ± s)

Group	n	VMAT2	DMT1	Nrf2
Normal group	20	15.98±0.70	6.56±0.64	15.22±1.22
Control group	24	8.45±0.59	12.76±0.86	8.57±0.54
Treatment group	30	11.69±1.17*△	9.13±0.44*△	12.46±0.62*△

Note：* $p < 0.05$ the treatment group vs the Normal group;△$p < 0.05$，the treatment group vs the control group

Discussion

The compound 6-OHDA may induce degenerative changes in specific catecholamine neurons, resulting in cell degeneration and necrosis of dopamine neurons. In 1968, Ungerstedt first reported a 6-OHDA-induced PD rat model (Ungerstedt 1968), which exhibited rotational behavior in response to APO (a DA receptor agonist); the rotation behavior could be quantitatively evaluated (Bove *et al.* 2005). Moreover, the model was economical, practical, and reliable and has been widely used in clinical practice and research. The present study showed that Bushenhuoxue Granules significantly reduced rotations and improved the rotational behavior of PD rats, thus providing an objective and reliable basis for PD treatment.

According to TCM, PD is one of the "tremor" conditions caused by liver and kidney deficiencies and is manifested as wind-phlegm stagnation and blood blockage. The treatment focuses on replenishing the kidney, invigorating the blood, dousing wind, and channeling meridians. Bushenhuoxue Granules are prepared on this principle: cistanche and dogwood replenish the kidney and essence and nourish the brain; Angelica, chuan xiong, and Polygonum invigorate the blood and dispel and eliminate stagnation; red peony and salvia invigorate the blood, disperse stagnation, alleviate pain, and prevent other drugs from harming yin by causing excessive warmth and dryness. Centipede channels wind, stops cramping, channels meridians, and alleviates pain; it "travels fast to internal organs and external meridians to open all places where qi and blood aggravate" and can carry other drugs to the brain. Hence, Bushenhuoxue Granules target both the cause and symptoms to replenish the kidney, nourish essence, invigorate the blood, and dispel stagnation. Previous clinical and experimental studies have confirmed that Bushenhuoxue Granules are effective (Yang *et al.* 2010, Li *et al.* 2012a, 2012b, Zhang *et al.* 2013, Yang *et al.* 2011, Yang *et al.* 2008, Yang *et al.* 2009a, 2009b, Wang *et al.* 2011, Li *et al.* 2011).

Pathologically, PD is characterized by the progressive loss of dopaminergic neurons in the pars compacta area of substantia nigra, the formation of Lewy bodies, and the ensuing decrease in striatal dopamine levels. PD is associated with many factors. Adenosine is an endogenous purine nucleoside and regulates the transmission of dopamine and other neurotransmitters in certain brain areas. It is closely related to the development of PD. A_{2A} receptor, an adenosine receptor (Latini and Pedata 2001), is controlled by γ-aminobutyric acid and acetylcholine and regulates the transmission of these two neurotransmitters. In the medium spiny neurons and olfactory tubercle of the striatum-globus pallidus area (Fink *et al.* 1992), the adenosine A_{2A} receptor and dopamine D2 receptor form heterodimers and/or heterologous oligomers to reduce the activity of the dopamine D2 receptor (Fuxe *et al.* 2005). The basal ganglion is the subcortex center of movement regulation and has both direct and indirect pathways. In the indirect pathway, efferent neurons contain mainly dopamine D2 receptor; thus, inhibition of the dopamine D2 receptor inhibits the indirect pathway. An imbalance of the direct and indirect pathways results in bradykinesia and tremor (Lang and Lozano 1998), whereas blocking the adenosine A_{2A} receptor will increase the activity of the dopamine D2 receptor, thereby treating PD (Ferré *et al.* 2002). Our previous study showed that Bushenhuoxue increases dopamine D2 receptor activity (Yang *et al.* 2011). The present study showed that adenosine A_{2A} receptor was expressed in the cytoplasm and cell membrane of the rat striatum, whereas adenosine A_{2A} receptor expression was not observed in the substantia nigra. Moreover, adenosine A_{2A} receptor expression was low in the normal group and was significantly increased in the control group, whereas expression in the treatment group was between the normal group and the control group, suggesting that Bushenhuoxue Granules decreased adenosine A_{2A} receptor expression in the rat striata in the treatment group but not to normal levels. Together, we conclude that Bushenhuoxue Granules can decrease adenosine A_{2A} receptor expression in the striata of PD rats, thus playing an adenosine A_{2A} receptor antagonist-like role and treating PD.

In the central nervous system, the uptake, storage, and release of monoamine neurotransmitters require specific transporters, such as VMAT. VMAT2 is a VMAT subtype and the only transporter that transports dopamine into the synapse for storage and release. Once synthesized in the neurons of the pars compacta of the substantia nigra, dopamine is taken up from the synaptic gap by the dopamine transporter, transported into small vesicles for storage by VMAT2 on the synaptic vesicle membranes, and then released into the synaptic gap for recycling (Zheng *et al.* 2006). VMAT2 removes toxins that enter vesicles via the dopamine transporter to reduce the damage to dopaminergic neurons (Miller *et al.* 1999a) and takes up monoamine neurotransmitters into synaptic vesicles to reduce the toxicity caused by free radicals produced by

the oxidation of monoamine neurotransmitters (Liu *et al.* 1992). VMAT2 dysfunction or a decrease in its expression reduces the number of dopamine transporters available for recycling, thus weakening the role of monoamine neurons in removing exogenous and endogenous toxins. As a result, exogenous and endogenous toxins accumulate in the cytoplasm, where they are degraded and inactivated by cytoplasmic monoamine oxidase to produce free radicals, aggravating the damage to neurons in the substantia nigra. The VMAT2 level is significantly reduced in the brains of PD patients, suggesting that a decrease in VMAT2 expression is closely related to the development of PD and that VMAT2 is a marker of PD damage (Miller *et al.* 1999b). The present study showed that VMAT2 expression in the rat striatum and substantia nigra was high in the normal group and was significantly decreased in the control group, whereas the expression in the treatment was between the normal group and the control group. These findings suggest that Bushenhuoxue Granules increase VMAT2 expression in the brain of PD rats, thus regulating the number of dopamine transporters that enter the synapse for storage, release, and recycling and promoting the removal of endogenous and exogenous toxins. These Granules thus play an anti-free radical role to reduce the damage to dopaminergic neurons.

Pathologically, PD is characterized by the progressive loss of dopaminergic neurons in the pars compacta area of substantia nigra,the formation of Lewy bodies, and the ensuing decrease in striatal dopamine levels. Iron deposition in the brain too much may lead damage to nerve cells, causing Parkinson's disease and other neurodegenerative diseases. In PD patients with brain iron accumulation in the substantia nigra pars(Sofic *et al.* 1991), can cause degeneration of dopaminergic neurons in the substantia nigra, induced alpha - synaptic nuclear protein aggregation(Sian-hulsmann *et al.* 2011). At present, the aggregation mechanism of iron in the substantia nigra is not very clear, which may be related to the abnormal expression of DMT1 in brain iron regulatory proteins. DMT1 is a transmembrane iron transport protein, participating in the absorption and metabolism of iron and other metal ions in the cell with high expression of in the substantia nigra(Salazar *et al.* 2008). The high expression of DMT1 in PD patients is the same as the abnormal aggregation of brain iron (Zhang *et al.* 2010). Studies have confirmed that there is a high expression of iron ions in the substantia nigra of Parkinson's disease mice co-existed with high expression of DMT1(Jiang *et al.* 2003). DMT1 is involved in the accumulation of the iron ion in substantia nigra, resulting in excessive oxidative stress, which contributes to the damage of dopaminergic neurons (Song *et al.* 2011), suggesting that DMT1 plays an important role in the pathogenesis of PD. DMT1 expression abnormalities can lead to iron metabolism disorders, abnormal aggregation, and ultimately lead to the occurrence of PD. This study founded that DMT1 protein was expressed in the striatum and substantia nigra of rats, and the expression of the normal group was less than that of the control group, which was increased remarkably, while the expression of the treatment group was between the above-two groups. The study indicated that although not reduced to the level of the normal group, DMT1 in the striatum and substantia nigra of the treatment group decreased. It might be inferred that Bushen Huoxue granule could reduce the DMT1 expression of PD model rats with the decrease of abnormal iron deposition in the brain, while degenerating oxidative stress, easing dopamine neurons damage and protecting the dopamine neurons, and thus contributing to the treatment of Parkinson's disease.

Nrf2 is a transcription factor protecting the tissue cells such as dopaminergic neurons(Cho *et al.* 2004), playing a leading role in the regulation of oxidative stress in the body. Nrf2 regulates the expression of antioxidant enzymes and proteins, easing oxidative stress damage, inhibiting the inflammatory response and serving anti apoptotic effects(Jain *et al.*2005, Giudice *et al.* 2010). Nrf2 and its cytoplasmic adaptor protein Keap1 are the central regulator of cellular oxidative stress response(Nguyen *et al.* 2009)，Nrf2's activity is precisely regulated by Keapl, in the physiological state, Nrf2 was concentrated in the cytoplasm with Keapl binding, and the activity was in the state of relative inhibition. When the organism is in the oxidative stress state, Nrf2 and Keap1were decoupled into the kernel and combined with the antioxidant response element sequence in the promoter region of the nucleus, which is responsible for the activation of the oxidative stress(Itoh *et al.* 1997). The neuroprotective effect of Nrf2 can also be realized by the overexpression of astrocytes in the striatum(Jakel *et al.* 2007). Under the situation of Nrf2 activation barrier or loss, the easing capacity of Nrf2 to oxidative stress is weakened. Consequently, the toxicity get enhanced, the dopaminergic neurons and astrocytes dysfunction ensues, even followed with apoptosis and death, and thus leading to the occurrence of PD. In this study, we found that Nrf2 protein was expressed in the striatum and substantia nigra of rats, while the expression of the normal group was more than that in the control group, which was significantly decreased, and the expression of the treatment group was between the two groups. We infer that the Bushen Huoxue granule can improve the expression of Nrf2 protein in the brain of PD model rats, playing the role of anti oxidative stress, inhabitation of the inflammatory reaction and anti apoptosis, protecting the dopaminergic neurons, so as to treat PD effectively.

Conclusions

The present study showed that Bushenhuoxue Granules improved the rotational behavior of PD rats, providing an objective and reliable basis for PD treatment. Bushenhuoxue Granules decreased adenosine A_{2A} receptor expression and increased VMAT2 expression, and we hypothesized that Bushenhuoxue Granules may play an adenosine A2A receptor antagonist-like role. Moreover, Bushenhuoxue Granules may regulate the number of dopamine transporters that enter the synapse for storage, release, and recycling and promote the removal of endogenous and exogenous toxins, thus playing an anti-free radical role to reduce the damage to dopaminergic neurons and treat PD. The present study showed that

Bushenhuoxue Granules improve the rotational behavior of PD rats, with the decreased the expression of DMT1 protein and the increase of Nrf2 protein in rat brain. We hypothesized that Bushenhuoxue Granules might reduce the abnormal deposition of iron in the brain, while functioning anti-oxidative stress, inhibiting inflammatory response, effecting as anti apoptosis, so as to protect the dopamine neurons.

Acknowledgments: This work was supported primarily by grant to Minghui Yang from the National Natural Science Foundation of China (81273969).

Conflict of Interest: The authors report no conflict of interest.

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